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Foxes As a Potential Wildlife Reservoir for *mecA*-Positive Staphylococci

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Abstract

Methicillin-resistant staphylococci (MRS), and methicillin-resistant *Staphylococcus aureus* (MRSA) in particular, have become a public and veterinary health concern. The search for MRS reservoirs outside human hospitals is needed in order to understand the reasons for their persistence and to control their spread. MRS have been isolated from rats, but little is known about their occurrence in foxes. In view of the perceived increasing proximity between people and foxes in the U.K. and the well-documented potential of foxes as hosts for zoonotic pathogens, this study examined whether foxes can be a reservoir for MRS. This study examined the carriage of staphylococci and their antimicrobial resistance patterns in 38 foxes (*Vulpes vulpes*) from rural and semirural areas in the U.K. Staphylococci were isolated by enrichment culture from nasal, oral, axillary, and perineal swabs and speciated by standard bacteriological tests and API ID32 STAPH (bioMérieux, Marcy l'Etoile, France). Antimicrobial resistance was investigated by disc diffusion tests and identification of *mecA*. Thirty-seven staphylococcal isolates were identified from 35 of the 38 foxes. All isolates were coagulase-negative and most frequently included species from the *S. sciuri* group (35%), *S. equorum* (27%), and *S. capitis* (22%). All were phenotypically resistant to methicillin, and *mecA* was detected in 33 (89%) of isolates, but only 10 (27%) showed broad β-lactam antibiotic resistance. Methicillin-resistant *S. aureus* was not identified. These results indicate that foxes are a potential wildlife reservoir for *mecA*-positive staphylococci. Selection pressure from environmental contamination with antimicrobials should be considered.

Key Words: Antimicrobial resistance—Foxes—*mecA*—Staphylococci—Wildlife.

Introduction

Antimicrobials are widely used for therapy and prevention of disease in human and veterinary medicine as well as in modern agriculture (Cabello 2006; Owens and Stoessel 2008). Expansive use has led to selection for antimicrobial resistance in bacteria, the spread of associated resistance genes, and to the emergence of multidrug-resistant pathogens that pose a potential threat to human health (Baquero et al. 2009; Tacconelli 2009; Heuer et al. 2011). Staphylococci, which colonize and infect humans and animals, and which can persist on environmental surfaces for several months, have been known for their potential to express antibiotic resistance since 1944 (Kirby 1944). Furthermore, they have been associated with multidrug-resistance since the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in human hospitals. Methicillin resistance has also been reported in up to 80% of clinical isolates of coagulase-negative staphylococci (CNS), and seems to be associated with concurrent resistance to non-β-lactam antimicrobial classes such as to aminoglycosides, macrolides, quinolones, and tetracyclines (Hope et al. 2008). Important examples of CNS in human medicine include *S. epidermidis, S. lugdugnensis,* and *S. saprophyticus,* which have emerged as the most common nosocomial pathogens isolated from bloodstream infections in several countries, and are frequently associated with implant- or catheter-related infections in immunocompromised people, or with endocarditis and urinary tract infections in immunocompetent hosts (Piette and Verschraegen 2008).

Resistance to methicillin is conferred by the *mecA* gene, which is integrated into the bacterial chromosome on a large mobile cassette element (SCC*mec*), and confers broad β-lactam antibiotic resistance via an altered penicillin-binding protein

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(De Lencastre et al. 2007). *mecA* in MRSA is thought to have been acquired by *S. aureus* through horizontal transfer from coagulase-negative species, and a wildlife origin for *mecA* has been proposed. Genetic homologues of the early *S. aureus* SCC*mec* elements have been identified in *S. sciuri*, and biochemical similarities between their penicillin-binding proteins have been demonstrated (Couto et al. 1996; Chambers 1997; Wu et al. 2001). The results from genetic analyses have since led to taxonomic changes, and due to the close relatedness of the staphylococcal species *S. sciuri*, *S. vituli*, and *S. lentus*, these three are now combined in the *S. sciuri* group. Members of this group are frequently found in rodents and other mammals, but also in humans (Stepanovic et al. 2005).

The search for MRS reservoirs outside human hospitals continues in order to understand and predict the epidemiology of antibiotic resistance, and to help limit the spread of methicillin-resistant pathogens. Companion animals and more recently pigs have been linked to recurrent MRSA outbreaks in human hospitals, and genetic analyses have implicated in-contact animals as vectors for human MRS acquisition (Voss et al. 2005; Loeffler and Lloyd 2010). Similarly, MRS have been identified from indoor and outdoor environmental sources (Scott et al. 2008; Soge et al. 2009).

However, little has been reported on the occurrence of MRS in wild animals, despite several reports highlighting examples of wildlife as reservoirs for antibiotic resistance. Antimicrobial resistance in staphylococci was higher in isolates from feral cats in the U.K. than from domestic cats (Patel et al. 1999). Rats on swine farms carried MRSA ST398, and were considered potential contributors to the spread of resistance within and between swine herds (van de Giessen et al. 2009). Multiple antimicrobial resistances were found in *Escherichia coli* isolated from small wild animals living in proximity to swine farms, and extended-spectrum β-lactamase-producing *E. coli* was isolated from foxes in central Europe (Literak et al. 1984; Kozak et al. 2009). Wild red-billed choughs (Family *Corvidae*) living in areas where manure land spreading is practiced showed high levels of multidrug resistance in their bacterial flora, in accord with resistance levels found in the manure (Blanco et al. 2009). Wild-living primates in contact with human refuse showed higher levels of resistant microflora compared to primates with little or no contact with humans (Rolland et al. 1985).

Following the dramatic expansion of red fox populations in some countries in Europe over the past decade, and the perceived closer proximity of foxes to humans (Gloor et al. 2001; Canid Specialist Group 2004), this study aimed to determine whether foxes should be considered a potential wildlife reservoir or vector for MRSA and other MRS.

Materials and Methods

Sampling of foxes

Thirty-eight dead foxes were sampled for staphylococcal carriage in the Scottish Borders and Pentlands between May and November 2007 and June and September 2008. The foxes were shot as part of pest-control programs in these areas, but the carcasses were retained for a Department for Environment, Food and Rural Affairs (DEFRA)-funded study into the role of carnivores as sentinels for emerging diseases being carried out at the Royal (Dick) School of Veterinary Studies. The animals were from rural areas except for four, which were found in semi-rural agricultural regions. They included male and female adult and subadult foxes, with some juveniles. The animals were sampled within 72 h of death.

Four sites were sampled per animal, including the nostrils (both with the same swab), buccal mucosa, axillary skin, and the perianal skin. The swabs were rolled over skin or mucosae for approximately 5 sec, and posted to the Royal Veterinary College in charcoal transport medium.

Swab processing

The four swabs from each animal were aseptically pooled in 10 mL tryptone soya broth (Oxoid, Basingstoke, Hampshire, U.K.) supplemented with sodium chloride (Sigma-Aldrich, Gillingham, Dorset, U.K.), to a total salt concentration of 10% for selective enrichment of staphylococci. After 24 h incubation at 37°C, the samples were streaked out onto 5% sheep blood agar (Oxoid), and presumptive staphylococci were identified based on salt resistance, colony morphology, Gram staining characteristics, and a positive catalase test. Up to three morphologically distinct staphylococcal colonies per sample were frozen at −80°C in brain heart infusion (Oxoid) with 20% glycerol (Sigma-Aldrich).

Staphylococcus *species identification*

Staphylococci were regrown from frozen and incubated at 37°C on blood agar for 24 h, and on mannitol salt agar (both Oxoid) and mannitol salt agar with 6 mg/L oxacillin (Sigma-Aldrich) for 48 h. Phenotypic tests for speciation included assessment of hemolysis, a slide coagulase test with dog plasma, and where negative, a tube coagulase test with rabbit plasma, DNase test, and acetoin fermentation (Voges-Proskauer reaction; Barrow and Feltham 1993). The API ID32 STAPH (bioMérieux, Marcy l'Etoile, France) identification kit was used for further speciation following the manufacturer's instructions. API strips were read manually and species identification was based on interpretation of scores by bioMérieux's online apiweb™ software.

Phenotypic resistance profiles

Methicillin resistance was screened for by growth on oxacillin-supplemented mannitol salt agar as described above, and agar color change from pink to yellow was indicative of mannitol fermentation. Antimicrobial resistance profiles were determined by disc diffusion tests on Mueller-Hinton agar following the Clinical and Laboratory Standards Institute protocol (The Clinical and Laboratory Standards Institute 2004) with colonies grown on blood agar. The following antimicrobial discs were used (Oxoid): methicillin (5 μg), ampicillin (10 μg), amoxicillin/clavulanic acid (30 μg), cefalexin (30 μg), clindamycin (2 μg), tetracycline or oxytetracycline (both 30 μg), trimethoprim-sulfamethoxazole (25 μg), enrofloxacin (5 μg), and fusidic acid (5 μg). Breakpoints were as defined by CLSI where available, for fusidic acid as defined by Olsson-Liljequist and associates (2002), and for cefalexin and enrofloxacin at 12 mm, in accordance with the manufacturers' recommendations. Multidrug-resistance was defined as resistance to three or more non-β-lactam antimicrobial classes (Merlino et al. 2002).

Genotypic resistance to methicillin

The presence of *mecA* was investigated in all isolates that were phenotypically resistant to methicillin by gene amplification through polymerase chain reaction (PCR) and subsequent gel electrophoresis. DNA was extracted from overnight cultures using a commercial purification kit (Bacterial Genomic DNA Purification kit; Edge Biosystems, Gaithersburg, MD) according to the manufacturer's instructions. Samples were prepared for PCR by adding 1 μL of extracted DNA to 49 μL PCR mix. The mix contained 5 μL 10 $\times$ PCR buffer, 1 μL 10 mM dNTP mix, 2 μL each of forward and reverse *mecA* primers, and 0.25 μL HotStarTaq DNA Polymerase (Qiagen, Crawley, West Sussex, U.K.). PCR primers and conditions were as described by Brakstad and colleagues (1992). A *S. aureus* MRSA ST398 *t*011 was used as *mecA*-positive control for all phenotypic tests and the PCR.

Results

Staphylococcus isolation and species identification

Thirty-five of the 38 foxes (92.1%) yielded staphylococci, and 37 different isolates were investigated. Two morphologically distinct staphylococcal isolates were identified from two foxes, and in both cases the two isolates represented two different species with distinct antimicrobial resistance patterns.

All isolates were coagulase-negative on both slide and tube coagulase testing. Two isolates were DNase-positive. These were also two of three isolates exhibiting β-hemolysis. Seven distinct staphylococcal species were identified by API ID32 STAPH. *S. sciuri* (35%), *S. equorum* (27%), and *S. capitis* (22%), were the most frequently identified species. Two isolates were identified as *S. chromogenes* (5%), two as *S. xylosus* (5%), one as *S. lentus* (3%), and another as *S. kloosi* (3%). All isolates were Voges-Proskauer negative, and these results matched the acetoin fermentation on API in all.

Antimicrobial resistance

All isolates grew on oxacillin-screening agar, and all showed resistance to methicillin on disc diffusion tests. High levels of resistance were seen, particularly to the other β-lactam antibiotics (Table 1), but only 10 isolates showed resistance to all four β-lactam antibiotics (Table 2). For non-β-lactam antibiotics, fusidic acid (19%) resistance was most frequently seen, followed by tetracycline (14%). Low frequencies of resistance (5%) were seen for enrofloxacin and clindamycin. None of the isolates were resistant to trimethoprim-sulfamethoxazole. Fourteen distinct resistance patterns were observed, and 84% of isolates were resistant to at least two of the four β-lactams, and 54% were resistant to at least three (Table 2). Most isolates (62%) were susceptible to all non-β-lactam antibiotics. In addition to β-lactam resistance, 32.4% showed resistance to one other class of antimicrobial, two isolates (5.4%) were resistant to two non-β-lactam antibiotics, while multidrug-resistance was not observed.

Table 1. Frequency of Resistance to Different Antimicrobial Agents Determined by Disc Diffusion Testing Among 37 Staphylococcal Isolates from Foxes

Class of antimicrobial	*Antimicrobial disc*	*Resistant isolates (%)*
β-Lactam		
Penicillin	Methicillin 5 μg	37 (100)
	Ampicillin 10 μg	25 (67.6)
	Amoxicillin/clavulanic acid 30 μg	19 (51.4)
Cephalosporin	Cefalexin 30 μg	17 (45.9)
Fluoroquinolone	Enrofloxacin 5 μg	2 (5.4)
Potentiated sulfonamide	Trimethoprim-sulfamethoxazole 25 μg	0 (0)
Tetracycline	Oxytetracycline and tetracycline 30 μg	5 (13.5)
Lincosamide	Clindamycin 2 μg	2 (5.4)
Sterol	Fusidic acid 5 μg	7 (18.9)

Table 2. Phenotypic Antimicrobial Resistance Patterns Among 37 Staphylococcal Isolates from Foxes

Resistance profiles	*Frequency (%)*
MET	3 (8.1)
MET, CL	6 (16.2)
MET, AMP	3 (8.1)
MET, TET	3 (8.1)
MET, CL, AMP	1 (2.7)
MET, AMC, AMP	4 (10.8)
MET, AMP, ENR	1 (2.7)
MET, AMP, DA	1 (2.7)
MET, CL, AMC, AMP	6 (16.2)
MET, AMC, AMP, TET	2 (5.4)
MET, AMC, AMP, FD	3 (8.1)
MET, CL, AMC, AMP, FD	2 (5.4)
MET, CL, AMC, AMP, ENR, FD	1 (2.7)
MET, CL, AMC, AMP, DA, FD	1 (2.7)

MET, methicillin; CL, cefalexin; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; TET, tetracycline; ENR, enrofloxacin; DA, clindamycin; FD, fusidic acid.

Presence of *mecA*

The presence of *mecA* was confirmed in 89% (n = 33) of phenotypically methicillin-resistant isolates. The four *mecA*-negative isolates did not appear to be associated with a specific species or disc resistance pattern. Three of those were resistant to all four β-lactam antibiotics including cefalexin, and the fourth showed resistance to three β-lactam antibiotics and tetracycline, but not cefalexin. Two of the *mecA*-negative isolates were DNase-positive, showed β-hemolysis, and were identified as *S. chromogenes* by API.

Discussion

The results from this study indicate that the staphylococcal microflora of foxes are a potential wildlife reservoir for *mecA*, a resistance gene of clinical significance in human medicine worldwide. Although a larger sample size and a more diverse geographical sampling area would have allowed statistical comparison between locations and proximity to people, to the authors' knowledge, this is currently the largest reported study on staphylococci and antimicrobial resistance in foxes. Foxes are well known vectors for zoonotic pathogens such as scabies mites and dermatophytes, but they have also been shown to carry mycobacteria (Millán et al. 2008), which may be associated with zoonotic spread and multidrug-resistance.

While all were negative for MRSA, the majority of foxes carried coagulase-negative MRS. High frequencies of coagulase-negative MRS have previously been reported in feral cats, and resistance had been attributed to environmental sources (Patel et al. 1999; Hariharan et al. 2011). In companion animals and people, coagulase-negative MRS had been isolated from between 20% and 82% of horses (Baptiste et al. 2005; Bagcgil et al. 2007; Loeffler et al. 2010), from 63% of their in-contact humans, and from 66% of stable environmental sites (Moodley and Guardabassi 2009). In contrast, only 7–15% of domestic dogs and cats were positive for coagulase-negative MRS in these screening studies. This may indicate that some hosts such as horses, humans, and now also foxes, support carriage of coagulase-negative MRS better than others, regardless of antimicrobial selective pressure.

All isolates in this study showed phenotypic resistance to methicillin, but broad-spectrum β-lactam resistance and *mecA* detection were not consistent in all. While procedural error cannot be excluded, similar findings have been reported in a CNS isolate (van Duijkeren et al. 2004), and most recently in the newly identified *mecA* (LGA151)-MRSA isolated from cattle in the U.K. and Denmark (García-Álvarez et al. 2011). Vigilance and a continuing effort to improve laboratory methods are warranted in order to avoid misidentification of important pathogens. The use of more discriminatory molecular methods for such isolates could improve confidence with regard to speciation, as even coagulase test results may be unreliable, as has been described for *S. (pseud)intermedius* (Cox et al. 1985; Zadoks and Watts 2009).

The CNS species identified from the foxes in this study and the predominance of the *S. sciuri* group reflect those commonly found in a wide variety of domestic and wild animals (Kloos et al. 1976; Kawano et al. 1996; Nagase et al. 2002; Devriese et al. 2009; Zhang et al. 2009). *S. sciuri*, *S. equorum*, *S. xylosus*, and *S. lentus* have all been isolated from the intestines of wild small animals such as shrews and voles (Hauschild 2001). *S. chromogenes* is associated with udder health problems in dairy cattle (De Vliegher et al. 2003). *S. capitis* is a common commensal of mainly human skin which has recently gained importance as an opportunistic pathogen, and *S. kloosii*, closely related to *S. sciuri*, is found mainly in wildlife (Schleifer et al. 1984; Takahashi et al. 1999). Members of the *S. sciuri* group are rarely found in humans and seldom cause disease, indicating that host-pathogen interactions and host specificity play a role in staphylococcal carriage (Irlinger 2008).

In conclusion, foxes appear to be competent carriers of several multi-host staphylococcal species and a potential source of the *mecA* gene. While the selection pressure on the microflora of foxes is likely to reflect a combination of naturally occurring and anthropogenic antimicrobial agents (Martinez 2008), a better understanding of their origin is needed to eventually limit the spread of resistance genes into pathogenic bacteria.

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Author Disclosure Statement

No competing financial interests exist.

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